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AMBER PARTICLES AS LIVING PLANT CELL MARKERS IN FLOW CYTOMETRY

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*The unique biological properties of amber are well known. Amber particles that penetrate into the cellular matrix can potentially be used as markers of plant cell biological activity by identification of living cells in flow cytometry. However, there have been no studies on effect of amber particles on plant cells. The aim of this study was to determine effect of amber nano- and micro- (5 nm^{-3} µm) particles on somatic and gametic cells and to assess the possibility to use amber particles as alive plant cells markers. To reach the aim, fluorescence of cells was determined in the presence of amber particles, and amber components — spirit of amber and sodium succinate dibasic hexahydrate. Cell fluorescence was measured using a BD FACSJazz® cell sorter for several plants species (*Argyranthemum frutescens, Cyclamen persicum, Hordeum vulgare and Linum usitatissimum*) with and without treatment of amber. Differences between a control cell group (without amber treatment) and treated cell group with amber particles depended on plant species. The presence of amber components (alcohol soluble amber fraction and sodium succinate) in cultivation media mostly had no influence on cell fluorescence. The study showed that amber particles (size 5 nm^{-3} µm) can be used as living plant cell markers, as the presence of amber particles in plant cell cultivation media resulted in substantially increased plant cell fluorescence in all investigated species, and there was no detrimental effect of amber particles on plant cells.*

Key words: Argyranthemum frutescens, Cyclamen persicum, Hordeum vulgare, Linum usitatissimum*, somatic cells, gametic cells, cell fluorescence.*

INTRODUCTION

Plants growing in urban areas are subject to the effects of pollutants that cause damage to plant cells. Flow cytometry (FCM) can be used to determine many cell parameters based on cell fluorescence, including cell ploidy and cell oxidative stress determination by estimation of peroxidase intensity (Djaković and Jovanović, 2003; Dožel *et al.*, 2007; Dimpka *et al.*, 2012). Cell fluorescence can be used to screen and diagnose early stages of infection in plants and changes in reinitiated cell culture. Changes in fluorescence can be an indicator of cell apoptosis (Martinez *et al.*, 2010; Carter *et al.*, 2013). FCM has several advantages: more than 20 parameters from each cell can be estimated by cell fluorescence changes, a large number of cells can be evaluated in a very short time, results can be statistically significant and whole populations of cells are represented (Dožel *et al.*, 2007; Bargmann and Birnbaum, 2009; Galbraith, 2010). Most available chemicals used as fluorescent dyes for flow cytometry are hazardous for cells, which causes problems in monitoring of living cell parameters' changes in a long time period. The finding of some nontoxic fluorescent substance for cell dying would give more opportunities to investigate reaction of living cells on changing environmental factors in controlled conditions using cell culture. In flow cytometry the resulting fluorescence is an arithmetical mean of fluorescence for a minimum of 1000 cells. For comparable and valid results it is very important that all investigated samples would contain only alive cells. Therefore, before plant cell cultivation, it is necessary to separate living plant cells from apoptotic cells, and from cells that are in the beginning of apoptosis. This is particularly important when the cultivatable cells are from plants that were grown in a contaminated environment and contain many damaged cells and tissues.

The unique biological properties of amber are well known. Amber particles that penetrate into the cellular matrix can potentially be used as markers of plant cell biological activity. This could be used to identify living cells for flow cy-

tometry. On the basis of previous studies (Lyashenko, 2014), amber was chosen in this study as a non-hazardous fluorescence enhancing substance (Neves-Petersen, 2010).

Amber is a fossilised resin mineral containing organic compounds. The largest world deposit of amber is located in the Baltic region. Baltic amber contains about 8% succinic acid e.g. butanedioic acid (alcohol soluble amber fraction), which is historically known as spirit of amber (Chambers, 1728). For centuries amber has been used in jewellery production and investigated in medicine. Many properties of succinate were discovered and documented as bioactive e.g. impacting living cells (Tretter *et al*., 1987; Suno *et al*., 1989; Knauf *et al*., 2006; Delhomme *et al*., 2009), for example, anxiolytic effects on animal (mouse) cells (Chen *et al.*, 2003). Succinic anhydride was successfully used to introduce a negative charge onto the surface of proteins that changed electron transfer kinetics, thus changing the proteins activity (Mie *et al.*, 2008). Succinic anhydride is also used in polysaccharide (hyaluronic acid) modification (Eenschooten, 2010). Succinic acid, known as butanedioic acid, is found in many organisms and is an important part of metabolic processes. A linear relationship was found between succinic acid production and enzyme activity (Bouche *et al.*, 2003; Agarwal *et al.*, 2007). An increase of succinic acid concentration in cells results in increase of the cell's self-fluorescence (Neves-Petersen, 2010).

In spite of well-known unique biological properties of amber, there were no studies on effect of amber on plant cells. The aim of the study was to determine changes of fluorescence in both somatic and gametic cells in presence of amber active (Lyashenko, 2014) nano-micro particles or amber components, like the alcohol soluble amber fraction and sodium succinate dibasic hexahydrate, as well as to assess the possible use of amber particles as living plant cells markers.

MATERIALS AND METHODS

Plant material. Plant material from four different plant species was used: flax (*Linum usitatissimum),* cyclamens (*Cyclamen persicum)*, barley (*Hordeum vulgare)*, and marguerite *(Argyranthemum frutescens)*. The species of plants were chosen from various genera. For experiments only fresh plant material was used. The plants were grown in greenhouse conditions in the Institute of Biology, University of Latvia and National Botanical Garden of Latvia. Somatic cells were obtained from flax (*Linum usitatissimum*) leaf calli culture. Flax calli culture was acquired using an earlier elaborated method (Grauda *et al.*, 2009; Grauda *et al.*, 2013). To obtain gametic cell cultures, immature microspore cells of cyclamens, barley and marguerite were used. Somatic cell culture and immature microspore cell culture preparation was based on the modified method of Kasha *et al.* (2003). The optimal stages of microspores were determined by a light microscope with magnification $\times 10^3$ (Barnabás, 2003). Plant's parts were collected and ground in a Waring Blender 8011 in 0.3 M of D-mannitol solution (pH 5.8). Each sample was ground in mode No. 2 up to five times for 20 seconds until a visually homogeneous suspension was obtained. Then all samples were filtered through mesh (50 μm) three times and the acquired liquid was collected in plastic 45 ml tubes. Samples were centrifuged (Eppendorf Centrifuge 5810R) at 4 °C, 900 rpm for 15 min. After centrifugation, the liquid phase was decanted and the cell sediment was washed with 45 ml 0.3 M D-mannitol solution and centrifuged again at 4 °C, 900 rpm for 15 min. 1 ml of acquired cell sediment contained about 600 000 cells (Kasha *et al.*, 2003). The liquid phase was poured off and 1 ml of cells were suspended in 4 ml liquid MS medium (Murashige and Skoog, 1962) (pH 5.8) and mixed. The cell culture quality was determined by light microscope magnification $\times 10^3$ before the start of cultivation and after 24 h of culture.

Preparation of amber particle suspension. A powder containing amber nano- and micro- (5 nm 3 μm) particles was prepared according to an earlier elaborated method (Lyashenko, 2014). Spirit of amber and sodium succinate dibasic hexahydrate were used in the research. To prepare a suspension of amber active particles, the powder was diluted in distilled autoclaved H_2O in concentration 1 mg/1ml and 10 μl of polyoxyethylene sorbitan monopalmitate emulsifier (Tween® 40) was added to the suspension. Tween® 40 is a non-ionic surfactant derived from sorbitan esters. Tween® 40 was used to prevent amber particles agglomeration. The suspension was stirred for 2 min until homogenous consistency.

Evaluation of effects of amber nano- and micro-particles and amber components. Separately for each plant species, 1 ml of prepared amber particles suspension was added to 10 ml of prepared cell suspension. The final concentration of amber particles in cell culture was approximately 0.1 mg/ml. The cell cultures were incubated in a speed shaking regime for 2 hours and for 24 hours. Cells incubated in the same conditions without amber treatment were used as a control group. To prepare the cell suspensions in sodium succinate dibasic hexahydrate the succinate was diluted in deionised autoclaved H_2O (1:1) and added to cell suspension in concentration 0.1 ml/1ml. Suspensions were also obtained by adding alcohol soluble amber fraction.

The experiments were performed at room temperature (23–25 °C). After incubation, the cell suspensions were filtered through a flow cytometry-pass filter (mesh 40 μm) before flow cytometer analysis.

Device and software used for measurement. A BD FACSJazz® cell sorter (BD Biosciences, USA) with flow cytometer function was used to measure fluorescence of plant cells. The device was equipped with a 100 μm nozzle and used phosphate-buffered saline (BD Pharmingen^{TM} PBS, BD Biosciences, USA) as a sheath fluid. Cell counting events were triggered by forward-scattered signal. The excitation of the cell fluorescence was made by a 488 nm Coherent Sapphire Solid State (blue) laser. The fluorescence emission was measured at 530 nm (bandwidth 30 nm) and 585 nm (bandwidth 29 nm). Before all the measurements,

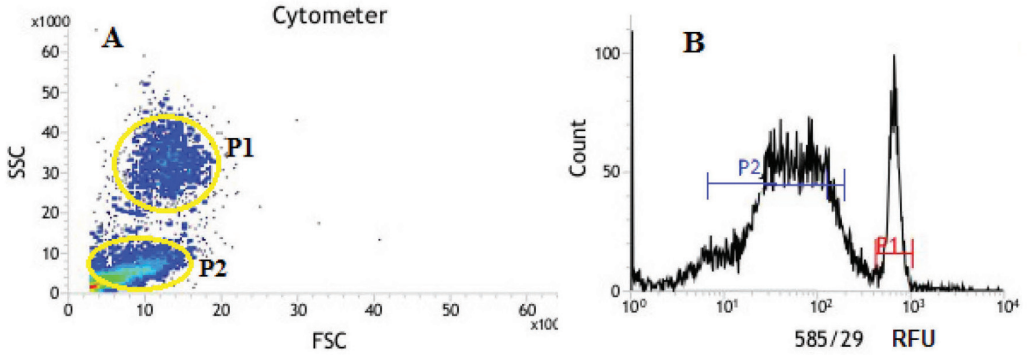

Fig. 1. **A.** Density plot of the fluorescence of barley (*Hordeum vulgare*) gametic cells cultivated in amber particle suspension (1 mg/ml) for 2 hours (P1) and suspended amber particles (P2) ; **B.** The fluorescence (RFU) in logarithmic scale at 585 nm for *Hordeum vulgare* gametic cells cultivated in amber particle suspension (1 mg/ml) for 2 hours (P1) and suspended amber particles (P2).

the flow cytometer was calibrated using Sphero^{TM} rainbow calibration particles (3.0 μm, BD Biosciences, USA) in phosphate buffered saline (PBS). The calibration was considered successful if the coefficient of variance (CV) did not exceed 3%. The intensity of cellulart fluorescence was expressed in arbitrary logarithmic units. No less than 5×10^3 gated cells from each sample were analysed.

The TDIST function (MS Excell) was applied to obtain the *p*-value. This value was used as a tool to test the null hypotheses at a certain level of significance. The significance threshold chosen was $p = 0.05$.

RESULTS

Altogether 142 samples were tested using flow cytometry. The established plant cell cultures contained more than 90% living cells, and a significant decrease of proportion of living cells after 24-h cultivation was not found. The fluorescence emission measured at 585 nm (bandwidth 29 nm) was much higher than fluorescence detected at 530 nm (bandwidth 30 nm), and therefore the results acquired from the 585 nm (bandwidth 29 nm) detection system were analysed. The amber particles had a rather wide (from about 10 till 700 relative fluorescence units (RFU)) range of self-fluorescence (Fig. 1). Nevertheless, the target cell group was clearly distinguishable. The difference in fluorescence intensity between cells cultivated with and without amber was statistically significant for all plant species cells using 2- and 24-hour incubation (Figs. 2–5). The flax somatic cells had lower fluorescence than gametic cells of all other tested plants: the fluorescence of the control somatic cell group (without amber particles) after 2-hour incubation was 155 RFU, the fluorescence of flax cells after 2-hour incubation with amber particles was 225 RFU and after 24-hour incubation — 255 RFU (Fig. 2). The presence of spirit of amber and sodium succinate in cultivation media did not have significant effect on flax somatic cell fluorescence.

The self-fluorescence of barley gametic cells after 2-hour incubation was 500 RFU and the addition of amber particles in cultivation media increased barley gametic cells fluorescence to 810 RFU after 2 hours and to 920 RFU after 24 hours of cell incubation. Cell incubation in media with alcohol soluble amber fraction and sodium succinate only slightly increased cell fluorescence (respectively 600 RFU after 2-h and 680 RFU after 24-h incubation) (Fig. 3). A rapid increase of gametic cell fluorescence after 2-hour cultivation in presence of amber particles was observed also

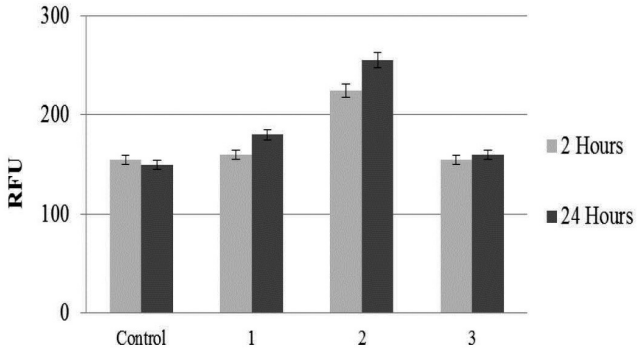

Fig. 2. Cell fluorescence (RFU) of flax (*Linum usitatissimum)* calli somatic cells at 585 nm after incubation at room temperature without and in presence of 0.1 ml/10 ml of alcohol soluble amber fraction (1), 0.1 mg/10 ml of amber particles (2) and 0.1 ml/10 ml of sodium succinate (3).

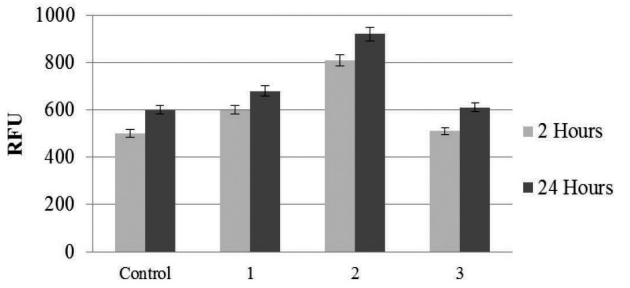

Fig. 3. Cell fluorescence (RFU) of barley (*Hordeum vulgare)* gametic cells at 585 nm after incubation at room temperature without and in presence of 0.1 ml/10 ml of alcohol soluble amber fraction (1), 0.1 mg/10 ml of amber particles (2) and 0.1 ml/10 ml of sodium succinate (3).

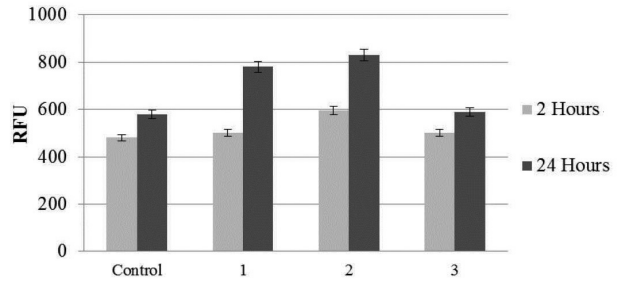

Fig. 4. Cell fluorescence (RFU) of marguerite (*Argyranthemum frutesgens)* gametic cells at 585 nm after incubation at room temperature without and in presence of 0.1 ml/10 ml of alcohol soluble amber fraction (1), 0.1 mg/10 ml of amber particles (2) and 0.1 ml/10 ml of sodium succinate (3).

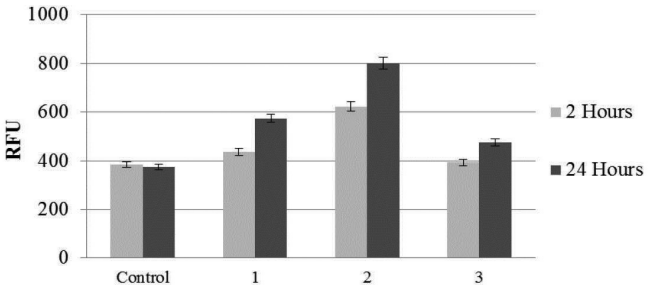

Fig. 5. Cell fluorescence (RFU) of cyclamens (*Cyclamen persicum*) gametic cells at 585 nm after incubation at room temperature without and in presence of 0.1 ml/10 ml of alcohol soluble amber fraction (1), 0.1 mg/10 ml of amber particles (2) and 0.1ml/10 ml of sodium succinate (3).

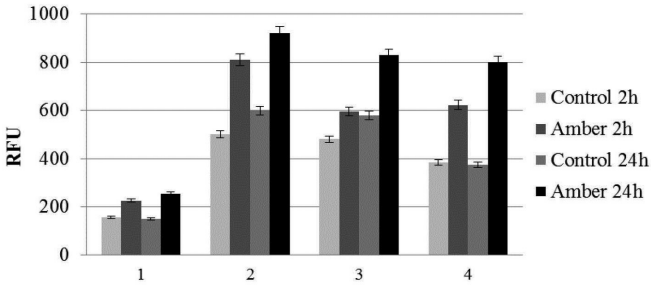

Fig. 6. Cell fluorescence (RFU) of gametic cells at 585 nm after incubation at room temperature without and in presence of 0.1 mg/10 ml of amber particles for both 2 and 24 hours: *Linum usitatissimum* (1), *Hordeum vulgare* (2), *Argyranthemum frutescens* (3), *Cyclamen persicum* (4).

for marguerite and cyclamens cells (Figs. 4 and 5). Addition of amber components (alcohol soluble amber fraction and sodium succinate) did not have significant effect on cell fluorescence after 2-h cultivation. Significant change of gametic cell fluorescence was observed after 24-h cell cultivation in media with the alcohol soluble amber fraction.

For all tested plant cells, incubation in media supplemented with amber particles resulted in considerably greater fluorescence after both 2- and 24-hour cultivation (Fig. 6). The difference between the control group and group treated with amber depended on plant species. For flax the difference in cell fluorescence after 2 hours of incubation was 45% (increase), which increased to 70% after 24-hour incubation. The highest fluorescence after incubation in the presence of amber particles was observed for gametic cells of barley: 62% increase after 2 hours of incubation and 53% increase after 24-hour incubation. Cyclamens showed the highest sensitivity to treatment with amber particles: 62% increase after 2 hours of incubation and 113% increase after 24-hour incubation.

DISCUSSION

Plant cells contain fluorescent pigments in different concentrations. Depending on species and cell specialisation, they contain fluorescent proteins in the chloroplasts, naturally fluorescent products such as proteins, including histones, and cell life process products, for example peroxidase (Neumann and Gabel, 2002; Kimura, 2005; Bargmann *et al.*, 2009; You *et al.*, 2015). All these factors together determine the cell self-fluorescence. In this study both somatic and gametic (young pollen) cells were used. Gametic cells were used in experiments to eliminate the fluorescence that could be produced by chloroplasts and by infected or apoptotic cells. Therefore, the observed increase of fluorescence of gametic cells could be linked to physiological state, and changes in relation to fluorescence of somatic cells reflects the sum of all physiological factors.

The tested plant cells reacted quite similarly to the amber components. The presence of amber components (alcohol soluble amber fraction and sodium succinate) in cultivation media mostly had no influence on cellular fluorescence. This indicates that the increase of cell fluorescence is not directly associated with the effect of succinic acid, a well-known phytohormone. The difference between the control cell group (without amber treatment) and group treated with amber particles depended on plant species. The observed difference in plant cell fluorescence may be genetical or reflect the physiological diversity of plant cells. Amber particles can enter cells only if they penetrate the cell membrane. This is mostly dependent on particle size, but also on particle polarity and chemical activity, as well as temperature, pressure and pH of the environment. Cellular excitation is most possible when amber particles are able to penetrate the cell membrane (Agarwal, 2007). The significant difference between fluorescence of cells cultivated in media with and without amber nano- and micro- (5 nm^{-3} μm) particles clearly shows that amber can affect plant cells. The difference in intensity of cell fluorescence after laser excitation of the investigated cells indicates specific properties of the cells and suggests the ubiquity of amber particles in cells. Plant cell wall pores have size 15 nm (Berestovsky *et al.*, 2001; Davison *et al.*, 2013). However, it should be noted that the cells from different plant species and with different function differ in cell wall architecture. The pore complex permeability of cells differs also, whereby much larger size molecules (particles) can enter living cells (Oparka *et al.*, 2004). As living plant cells have some surface potential (Kinrade *et al.*, 1998; Wang *et al.*, 2008) and amber particles are charged as well, amber particles may be bound to the surface of living cells (in case of opposite charges). In our opinion the observed substantial increase in cell fluorescence was likely due to both of these factors.

The study showed that it is possible to use amber particles (size 5 nm^{-3} μm) as living plant cells markers — the presence of amber particles in plant cell cultivation media resulted in increased plant cell fluorescence of all investigated species, and there was no detrimental effect of amber particles on plant cells.

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DZINTARA DAĻIŅAS KĀ DZĪVU AUGU ŠŪNU MARĶIERI PLŪSMAS CITOMETRIJĀ

Labi zināma ir dzintara labvēlīgā ietekme uz dažādiem organismiem, tomēr ir ļoti maz pētījumu par dzintara ietekmi uz augu šūnām. Tādēļ šī pētījuma mērķis bija noskaidrot nano un mikro lieluma (5 nm^{-3} μm) dzintara daļiņu un dzintara komponentu (spirtā šķīstošā dzintara frakcija un sukcināta) ietekmi uz augu somatiskajām un gametiskajām šūnām, un noskaidrot dzintara daļiņu piemērotību dzīvu augu šūnu iezīmēšanai urbānās vides pētījumiem, izmantojot plūsmas citometriju. Relatīvā fluorescence, izmantojot BD FACSJazz® šūnu šķirotāju ar plūsmas citometra funkciju, tika noteikta četru dažādu ģinšu sugu (*Argyranthemum frutescens*, *Cyclamen persicum, Hordeum vulgare, Linum usitatissimum)* augu šūnām pēc 2 un 24 stundu kultivācijas barotnēs ar un bez dzintara daļiņām vai dzintara komponentiem. Konstatēts, ka dzintara daļiņu klātbūtne visām pētīto augu šūnām ievērojami palielināja relatīvo fluorescenci. Dzintara komponentu ietekme uz šūnu relatīvās fluorescences izmaiņām nebija būtiska, salīdzinot ar dzintara daļiņu ietekmi. Dzintara daļiņas nesamazināja dzīvo šūnu daudzumu paraugos. Konstatēts, ka dzintara daļiņas (5 nm^{-3} μm) var izmantot kā marķierus dzīvo šūnu iezīmēšanai plūsmas citometrijas pētījumos.